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Chemistry

Increasing the chemical potential of the germ-line antibody repertoire

(coordination complex/catalysis/cofactors/transition metals/transgenic mice)

NORA SARVETNICK*, DEEPAK GURUSHANTHAIAH*, NIANHE HAN†, JAMES PRUDENT‡, PETER SCHULTZ‡, AND RICHARD LERNER†

Departments of *Neuropharmacology and †Chemistry and Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037; and ‡Department of Chemistry, University of California, Berkeley, CA 94720

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ABSTRACT **To augment the chemical potential of the immunological repertoire, a metal ion-binding light chain has been introduced into the murine genome. Mice containing the transgene were subsequently immunized with a fluorescein conjugate. The transgenic light chain was found at a high frequency in the anti-fluorescein memory B-cell compartment. This general method should be applicable to other cofactors and small molecules and should lead to generation of antibodies with unique catalytic activities.**

The humoral immune response provides a mechanism for evolving receptors specific either for stable molecules or for highly energetic transition states. The latter antibodies have been shown to act as selective chemical catalysts and share many properties with enzymes (1). Moreover, catalytic antibodies accelerate reactions by many of the same mechanisms used by enzymes, including approximation of reactants, general acid–base catalysis, and covalent catalysis (2). However, in order to extend the chemical potential of the immune system one would like to introduce into the immunological repertoire catalytic functionality beyond the side chains of the 20 natural amino acids.

In principle, catalytic cofactors can be introduced into an antibody in a number of ways—as an element of the hapten used in the immunization (3) or by directed mutagenesis of an existing antibody to create a cofactor binding site (4). Perhaps even more powerful is an *in vivo* approach that takes advantage of the two-chain nature of the antibody molecule to introduce the catalytic group and the mammalian immune system to select high-affinity antibodies. For example, a cofactor-binding light chain, introduced transgenically into mice, should assemble with endogenous murine heavy chains during the immune response to directly produce antibodies with unique chemical activities. Previous experiments with immunoglobulin light-chain transgenic mice have demonstrated that the exogenous sequences can be expressed in the B-cell compartment and that the transgene can dominate the antibody repertoire due to inhibition of rearrangement of the endogenous alleles (5–7). Importantly, the light-chain transgenes have also been shown to undergo somatic hypermutation (8). Thus, the introduced metal-binding light chain could shift the murine repertoire to contain predominantly cofactor-binding specificities that could be selectively refined by immunization. Herein, we report the introduction into the murine genome of an immunoglobulin light-chain gene that encodes a protein previously shown to bind the electrophilic cofactors Zn(II) and Cu(II) when part of an antibody molecule (4). Immunization studies showed that this light chain participates frequently in the immune response induced to a small organic hapten.

MATERIALS AND METHODS

Construction of Transgene and Transgenic Mice. The plasmid pB-14 (5, 6) was obtained from U. Storb (University of Chicago). To derive the plasmid used for microinjection, pB-14 was cut with *Eco*RI (blunt)/*Pvu* I (blunt), and a 1.8-kb fragment corresponding to the 5′ regulatory region and variable domain was subcloned into pBS-SKII cut with *Sac* I (blunt)/*Hin*cII. A natural *Sac* I site within the subcloned κ sequences was removed by digestion with *Sac* I, blunt-ending, and self-ligation. To insert the metal-binding light-chain sequences, a *Sac* I site was then introduced into the second exon of the MOPC21 κ-chain gene by site-directed mutagenesis with the oligonucleotide 5′-AGATTGGGT-CATTACGGCCG TCGACGGATG AGCTCAATGT TC-CCATCAGC-3′, containing the restriction sites *Eag* I, *Hin*cII, and *Sac* I (boldface). The resulting plasmid was cut with *Sac* I/*Hin*cII and ligated with the metallo-light-chain QM212 from a donor plasmid cleaved with *Xba* I (blunt)/*Sac* I. The resulting plasmid was then cut with *Eag* I (blunt)/*Xho* I and ligated with the 12-kb *Pvu* I (blunt)/*Xho* I fragment from pB14 that contained the enhancer and terminator regions. The final construct was cut with *Bss*HI to remove the plasmid sequences, and the 14.5-kb insert was purified for microinjection.

Transgenic mice were produced by standard techniques using mice of the (C57BL/6 × BALB/c)F_2 genetic background. The presence of the transgene was initially verified by Southern blotting using the QM212 insert as a probe and thereafter by PCR analysis. The mice were maintained by backcrossing to the BALB/c strain.

RNA Isolation and PCR Analysis. Flash-frozen tissue was homogenized in 7.6 M guanidine hydrochloride/50 mM potassium acetate and EtOH precipitated overnight at −20°C. RNA was recovered by centrifugation, resuspended in guanidine hydrochloride, and EtOH precipitated for 3 hr. RNA was again recovered by centrifugation, resuspended in a small volume of guanidine hydrochloride, extracted with phenol/chloroform (1:1), and EtOH was added; RNA was precipitated again, washed with 70% EtOH, resuspended in 10 mM EDTA, and stored at −70°C. A reverse transcriptase kit (GIBCO/BRL) was used for cDNA production from the purified RNA. PCRs were performed in an Ericomp (San Diego) twin block cycler. Plasmid and genomic DNA were amplified as follows: 94°C for 1 min 30 sec, 25 cycles of 94°C for 30 sec, 60°C for 45 sec, 72°C for 45 sec, and 72°C for 45 sec. For cDNA, the annealing temperature was 66°C and the extension time was 1 min 15 sec. The final extension time was

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Abbreviation: FITC–BSA, fluorescein isothiocyanate conjugated to bovine serum albumin.

extended to 5 min at the same temperature. For transcriptional analysis of transgenic mice, the oligonucleotides used were 5′-ATGCATCAGACCAGCATGGGC-3′ and 5′-CACTCTGACCATCAGCAGTGTGCA-3′.

Immunization and Hybridoma Production. Mice were immunized with fluorescein isothiocyanate conjugated to bovine serum albumin (FITC–BSA) in RIBI adjuvant system (RAS). The mice initially received 150 μg of FITC–BSA subcutaneously. This dose was repeated after 3 weeks, followed in 2 weeks by injecting with 100 μg of FITC–BSA intraperitoneally. One month later, a final injection of 50 μg of FITC–BSA was given, without adjuvant, by tail vein injection. On the 3rd day after the final injection, spleen cells were used for fusion to produce anti-fluorescein hybridomas by standard techniques.

Isoelectric Focusing. Isoelectric focusing of the antibodies was accomplished by using Novex (San Diego) isoelectric focusing gels (pH 3–10) and the accompanying buffers. Transblotting was done on a Millipore Immobilon-P poly(vinylidene difluoride) transfer membrane. Ascites was diluted to achieve a concentration of 10 ng/μl before reducing with 2-mercaptoethanol. Ten microliters of dilution combined with 10 μl of Novex 2× sample buffer were incubated at room temperature for 15 min. The samples were then loaded on the gel and run in a Novex vertical electrophoresis apparatus at 2 W per gel for 2 hr. After the run, gels were soaked in carbonate transfer buffer before transblotting for 1.6 hr at 150 mA. Blocking was accomplished with 10% nonfat dried milk in 1× Tris-buffered saline (TBS)/0.05% Tween 20 overnight. Alkaline phosphatase-conjugated mouse anti-κ antibody (Caltag, South San Francisco) was used at 1:1000 and color development followed with the AP color development kit (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) (Bio-Rad). Washes between blocking and incubations were done with 1× TBS/0.05% Tween 20.

RESULTS

A three-histidine metal ion coordination site with specificity for Cu(II) and Zn(II) had previously been introduced into the light chain of the fluorescein-specific antibody 4-4-20 (4). To express this metallo-light chain *in vivo*, the variable and constant region coding sequences were embedded into the second exon of the MOPC21 κ-chain gene, since this gene had been demonstrated to elicit allelic exclusion in transgenic mice (5, 6). The resulting light-chain molecule contained the MOPC21 leader peptide fused to our metallo-light chain variable and constant region domains (Fig. 1). The DNA construct was microinjected into fertilized zygotes. Twenty-one founder generation progeny were screened for integration of the transgene by Southern hybridization of genomic DNA and four lines of transgenic mice were identified. Three of these lines were propagated for further analysis.

To determine whether the transgene was expressed in lymphoid tissue, sets of PCR primers were designed that distinguish between the transgene and endogenous light-chain mRNAs (see Fig. 1). The primer specificities were confirmed by PCR analysis of both the injected plasmid and cDNA from nontransgenic mice. Since the transgene consists of metallo-light-chain sequences inserted into κ-chain genomic sequences, the transgenic transcript should be ≈400 bp longer than the endogenous transcript. Amplification of cDNA from two transgenic mice, 1853 and 1873, yielded an 850-bp fragment, while in nontransgenic mouse 1908 and BALB/c mice a 400-bp fragment was observed (Fig. 2). The size of the PCR product in the transgenic mice corresponds to the predicted length of the transgenic transcript. PCR analysis of genomic DNA from the same mice yielded a fragment of ≈1150 bp. The increase in size of DNA as compared with RNA (cDNA) corresponds to the length of the first intron, indicating that the transgenic transcript was correctly spliced. The structure of the transgenic transcript in this region was confirmed with additional primer sets (data not shown). Further studies demonstrated that all three transgenic lines expressed the transgene, although the level was somewhat higher in one line when assessed in comparison with actin primer standards. We also studied nonlymphoid tissue for expression of the transgene and detected only very faint bands on our PCR gels, which probably owe their origin to the presence of blood lymphocytes in all tissues (Fig. 2). This provides evidence for tissue-specific expression of our construct in the transgenic mice.

To determine whether the metallo-light chain could participate in an antigen-driven immune response *in vivo*, immunized mice were studied. Since the metallo-light chain was derived from a single chain anti-fluorescein antibody (9), we surmised that the chain might be used with reasonable frequency to produce anti-fluorescein antibodies. The murine anti-fluorescein response is known to exhibit significant affinity maturation and is quite diverse in that there appears to be no dominant idiotype (10). The participation of the transgene in the anti-fluorescein response would require selection into the memory B-cell compartment, resulting in incorporation of the metallo-light chain into high-affinity antibody molecules. Transgenic and nontransgenic mice from line 1465 were immunized with fluorescein. Both transgenic and nontransgenic mice produced comparable high-titer anti-fluorescein responses. Six hybridoma lines that bound to fluorescein were generated from the immunized transgenic mice (designated H1–H6). To determine whether any of these hybridomas expressed the transgene, RNA was extracted and cDNA was prepared and analyzed by PCR methods as described above. Of six hybridomas examined, two (H3 and H6) contained the transgenic transcript (Fig. 2). The other four hybridomas also contained the transgene genomic DNA but harbored no corresponding transcript. Due to the significant affinity maturation observed in the anti-fluorescein response, we determined whether the metal coordinating histidines were altered by somatic mutation in our hybridomas. DNA sequence analysis indicated that the κ chains of the hybridomas retained the metal-coordinating histidine residues.

Transgene RNA expression studies were confirmed by analyzing the corresponding immunoglobulins. Western blot analysis confirmed that both hybidomas contained κ chains. The supernatants from the hybridomas were reduced, separated by one-dimensional isoelectric focusing, and blotted with an anti-κ-chain-specific antibody. Hybridomas H3 and H6 demonstrated a unique band corresponding to an 8.6–9.3

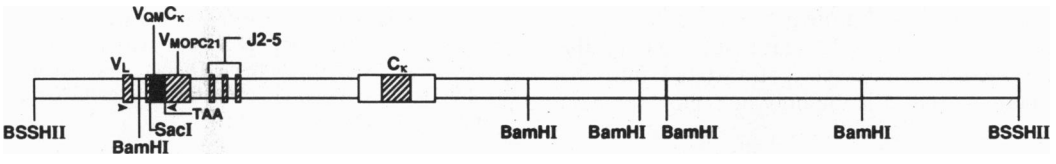

FIG. 1. Schematic representation of the transgene. After site-directed mutagenesis of pB14, the QM212 coding sequences were inserted into the second exon. Hatched boxes, κ-chain gene exons from pB14; solid box, inserted QM212 sequences. Arrowheads indicate position of PCR primers (ATG/*Pvu*) used in analysis of transgenic mice below.

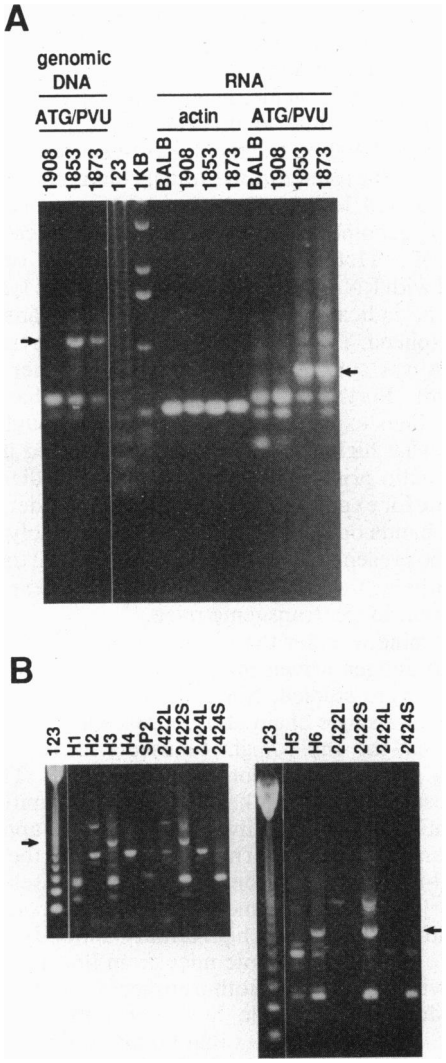

FIG. 2. Expression of transgene sequences in tissue and hybridomas. Ethidium bromide-stained agarose gel of PCR. (*A*) (*Left*) PCR analysis of tail genomic DNA with primers (ATG/*Pvu*) flanking the inserted QM212 sequences (see Fig. 1). DNA from a nontransgenic mouse (lane 1908) contains a single band, whereas DNA from transgenic mice (lanes 1853 and 1873) contain a second band (arrow) corresponding to the QM212-containing transgene. (*Right*) PCR analysis of cDNA derived from blood RNA from nontransgenic (lanes BALB and 1908) and transgenic (lanes 1853 and 1873) mice. Equivalent bands are seen with primers for actin. As indicated from the ATG/*Pvu* primers, all mice contain the band corresponding to the spliced transcript from the endogenous κ-chain gene, and the transgenic mice contain an additional band (arrow) corresponding to spliced transcripts from the transgene. (*B*) PCR analysis of hybridomas H1–H6 derived from spleen cells of FITC–BSA immunized transgenic mice. cDNA prepared from RNA extracted from the hybridomas or from the liver (L) and spleen (S) from transgenic (lanes 2422) and nontransgenic (lanes 2424) mice were amplified with the ATG/*Pvu* primers. Band corresponding to the transgene transcript (arrow) was seen in hybridomas H3 and H6 in addition to the spleen of transgenic mouse 2422.

isoelectric focusing point that is quite near the 8.8 theoretical pI of the transgenic κ chain (Fig. 3). This result supports the notion that the κ-chain transcript is translated into the metallo-light chain and that it is the only light chain present in these hybridomas.

DISCUSSION

Our results demonstrate the power of transgenic technology for *in vivo* generation of antibodies that contain cofactors for

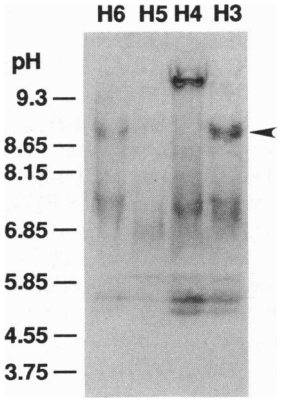

FIG. 3. Western blot of isolectric focusing gel. Protein G purified ascites from hybridomas H6, H5, H4, and H3 were reduced, separated on an isoelectric focusing gel, blotted, and reacted with an anti-κ-chain antibody. Hybridomas H6 and H3 contain a unique band corresponding to an isoelectic focusing point of pH 8.6–9.3.

catalysis. In essence, the chemical potential of the antibody repertoire has been vastly increased. The transgenic light chain was found at a high frequency in the anti-fluorescein memory B-cell compartment. This general method could be extended to other antigens and other light chains. For production of catalytic antibodies to antigens with multiple epitopes, such as viral antigens, our *in vivo* approach would select for responsiveness to epitopes of highest accessibility and immunogenicity and, thus, may be useful for deriving therapeutic antibodies.

Although previous studies have shown that transgenic light chains can participate in the humoral response to multiple antigens (7), the response to some antigens may be diminished due to the elimination or inactivation of self-reactive B cells (11–14). Several genetic approaches can be used to broaden the available B-cell repertoire. For example, we have introduced the transgene into Ipr mice, which produce autoantibodies due to a defect in the *Fas* gene, which is involved in programmed cell death (15, 16). Similarly, we anticipate that the ectopic expression of the *Bcl-2* gene, which promotes B-cell survival (17–19), would increase the available B-cell repertoire. Use of mice deficient in endogenous κ chains for transgenic experiments should eliminate background and facilitate screening a greater number of catalytic antibody molecules.

The ability to augment the murine immunological repertoire with cofactors or other small molecules significantly expands the chemical capacity of the antibody molecule. This general method can be extended to include other metal ion coordination sites (20), other cofactors such as flavins (21), and perhaps even cytotoxic or imaging agents. Finally, the ability to recombine a given metallo-light chain *in vitro* with a large number of heavy chains using combinatorial strategies should expand the potential of this approach even further (22).

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